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Extraction of Sulfur Mustard Metabolites from Urine Samples and Analysis by Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS)

B. P. Mayer, A. M. Williams, R. N. Leif, R. N. Udey, A. K. Vu

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Disclaimer

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Title: **Extraction of Sulfur Mustard Metabolites from Urine Samples and Analysis by Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS)**

Prepared by: Brian P. Mayer, Audrey M. Williams, Roald N. Leif, Ruth N. Udey, and Alexander K. Vu

Prepared at: Forensic Science Center
Lawrence Livermore National Laboratory
mayer22@llnl.gov, 925-423-1128

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Introductory Notes/Comments:

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Approval, Concurrence or Review:	Name & Title/Position	Date
☑ Approval ☐ Review ☐ Concurrence	Audrey Williams, Audrey Williams, Lakeshore PI	08.26.14
☐ Approval ☐ Review ☐ Concurrence		
☐ Approval ☐ Review ☐ Concurrence		
☐ Approval ☐ Review ☐ Concurrence		
☐ Approval ☐ Review ☐ Concurrence		

1. SCOPE AND APPLICATION:

The following procedure describes the extraction of sulfur mustard metabolites (principally 1,1'-sulfonylbis[2-(methylthio)ethane] (SBMTE)) and related chemicals from urine and subsequent analysis by liquid chromatography with detection by high resolution mass spectrometry (LC-HRMS) to determine signatures present in a contaminated urine sample. Positive identification of such signatures in urine may signify patient exposure to sulfur mustard (HD). A future SOP will detail analysis of these metabolites by gas chromatography-tandem mass spectrometry (GC-MS/MS).

2. SUMMARY OF METHOD:

Urine samples containing SBMTE (and partially or fully oxidized versions) and thiodiglycol (TDG) are processed using Solid Phase Extraction (SPE) to extract and preconcentrate the analytes from the urine matrix. The final extract is then analyzed by LC-HRMS techniques to detect and quantify the analytes of interest.

3. INTERFERENCES:

3.1. Urine metabolites – amino acids, biological metabolites
3.2. Protein, blood, foreign contaminants
3.3. Sample preparation contaminants (titanium dioxide, unreacted titanium trichloride, etc.)

4. SAFETY:

4.1. This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA regulations regarding the safe handling of the chemicals listed in this method. A reference file of material safety data sheets (MSDSs) should be available to all personnel involved in these analyses.
4.2. Urine is a biological matrix and as such, much be handled under appropriate BSL-1 conditions. It is recommended that an unknown urine sample be handled at BSL-2 conditions until it is determined that no BSL-2 substances are present in the sample (e.g. blood).
4.3. Standard precautionary measures should be used for handling all solvents included in this method. Suspect urine samples should be treated as if they contain a high concentration of sulfur mustard and related metabolites until it is proven otherwise.

5. APPARATUS:

5.1. Agilent 1290 Infinity HPLC coupled to an Agilent 6530 Q-TOF LC/MS detector with a Dual Agilent Jet Stream Electrospray Ionization source
5.2. Agilent MassHunter Data Acquisition software (version B.05.01) and Q-TOF Quantitative Analysis software (version B.05.00)

6. REAGENTS AND MATERIALS:

6.1. ChemElut Diatomaceous Earth (3 mL, unbuffered) cartridges.(Agilent, 12198003)
6.2. Methanol, HPLC Grade.
6.3. Titanium(III) trichloride ($TiCl_3$) (10 wt% in 20-30 wt% HCl)
6.4. Pooled human urine (Innovative Research)
6.5. Native and deuterated SBMTE (synthesized in-house)
6.6. Formic Acid, LC/MS-Grade (Fisher, A117)
6.7. Acetonitrile, LC/MS-Grade (Fisher, A995)
6.8. Water, 18 MΩ from Millipore Milli-Q Advantage A10 system
6.9. Autosampler Vials (Phenomenex, AR0-9923-13), 250 µL Inserts (Agilent, 5181-8872), and Caps (Agilent, 5182-0723)
6.10. Atlantis T3 C18 column, 100 Å pores, 3 µm particles, 2.1 mm x 150 mm (Waters, 186003719)

7. SAMPLE COLLECTION, PRESERVATION AND HANDLING:

Samples should be frozen at -80°C as soon as possible after collection and brought slowly to room temperature immediately before analysis. Method development samples are spiked with the protonated and deuterated SBMTE in-house. All handling and transportation will be in accordance with the LLNL chemical warfare agent and BSL-2 safety procedures.

8. STANDARDS AND CONTROLS:

8.1. Native 1,1'-sulfonylbis[2-(methylthio)ethane] (SBMTE)
8.2. Stable isotope labeled SBMTE as an internal standard: 1,1'-sulfonylbis[2-(d_3-methylthio)ethane] (D6-SBMTE)

9. CALIBRATION:

9.1. ESI-TOF Calibrant, ESI – L Low Concentration Tuning Mix (Agilent, G1969-85000)
9.2. API-TOF Reference Mass Solution Kit containing purine and HP-0921 (Agilent, G1969-85001)

10. SAMPLING:

This method was developed using pooled human urine samples prepared by the following spiking procedure:

10.1. Aliquot a known volume of urine into a glass vial.
10.2. Known amount of SBMTE was spiked in the urine sample.
10.3. Sample vortexed to thoroughly combine sample before extraction.

11. OTHER QUALITY ASSURANCE CONSIDERATIONS:

A derivatization reagent blank should be run with each set of samples, as well as a method blank, if an uncontaminated urine sample is available. Samples should be extracted and analyzed in triplicate, if enough sample is obtained.

12. PROCEDURE (Step-by-Step Directions)

12.1. Preparation of Calibration Standards

12.1.1. Prepare separately two working stock solutions, one of the SBMTE and one of the deuterated internal standard D6-SBMTE, in 5% acetonitrile in Milli-Q water.

12.1.2. Using individual vials with inserts, prepare a 7-point standard curve of SBMTE bracketing the expected analytical range [e.g. 1, 5, 10, 50, 100, 175, 200 ppb] by adding appropriate amounts of SBMTE working stock.

12.1.3. Add the same amount of D6-SBMTE internal standard to each individual calibration standard (e.g. 100 ppb).

12.1.4. Vortex and analyze directly using LC-MS.

12.2. Sample Preparation

12.2.1. Add D6-SBMTE internal standard mixture to a clean vial.

12.2.2. Evaporate the solvent.

12.2.3. Add 0.5 mL of urine sample and vortex for 60 seconds.

12.2.4. Add 1 mL of $TiCl_3$ (10% in 30% HCl) solution to sample.

12.2.5. Vortex for 1 minute and heat sample at 75°C for no more than one hour.

12.2.6. Allow sample to cool to room temperature.

12.2.7. Add 2 mL of 6 N NaOH. Allow sample to sit at room temperature overnight. Liquid will be clear in the next day.

12.2.8. Centrifuge at 4000 rpm for 10 minutes. Decant supernatant into a new 15 mL centrifuge tube.

12.2.9. Centrifuge at 4000 rpm for 5 minutes. Decant supernatant directly onto an unconditioned Varian ChemElut Diatomaceous Earth cartridge.

12.3. Solid Phase Extraction (SPE) procedure is as follows:

12.3.1. Cartridge (Agilent ChemElut, 3ml, unbuffered)

12.3.1.1. Sample added to unconditioned cartridge.

12.3.1.2. Collect all eluant using 4 x 4mL of a solution of DCM/acetonitrile (60:40).

12.3.1.3. Dry captured eluant to complete dryness by heating sample at 40°C and evaporating using a gentle stream of nitrogen.

12.3.1.4. Reconstitute in 250µl of 5% acetonitrile in water and vortex for 1 minute.

12.3.1.5. Transfer 125µl into autosampler insert and vial for LC-MS analysis.

12.4. Instrument Conditions for LC-HRMS analysis are as follows:

12.4.1. Injection volume: 10 µL

12.4.2. Mobile phase solvent A: 0.1% formic acid in Milli-Q water

12.4.3. Mobile phase solvent B: 0.1% formic acid in LC/MS-Grade acetonitrile

12.4.4. Mobile phase gradient program:

12.4.4.1. 95% solvent A for 5 minutes

12.4.4.2. Linear gradient to 10% solvent A over 15 minutes

12.4.4.3. 95% solvent A for 10 minutes (column re-equilibration)

12.4.4.4. Total analysis time: 30 minutes

12.4.5. Mobile phase flow rate: 0.5 mL/min

12.4.6. Column temperature: 45 °C

12.4.7. Ionization: electrospray, positive mode

12.4.8. Capillary voltage: 5000 V

12.4.9. Nozzle voltage: 900 V

12.4.10. Source gas temperature: 320 °C

12.4.11. Source gas flow rate: 4 L/min

12.4.12. Nebulizer: 55 psig

12.4.13. Sheath gas temperature: 250 °C

12.4.14. Sheath gas flow rate: 6 L/min

12.4.15. Mass scan range: 50-1000 *m/z*

12.4.16. Scan rate: 3 spectra/second

12.4.17. Masses extracted for data analysis:

Compound	**Formula**	**$[M+NH_4]^+$**
SBMTE	$C_6H_{14}O_2S_3$	232.0494 ± 20 ppm
D6-SBMTE	$C_6H_8D_6O_2S_3$	238.0871 ± 20 ppm

12.5. Run each sample by aliquotting for direct injection:

12.5.1. All LC samples can be directly injected.

12.5.2. Run the derivatization reagent blank before running the calibration samples and *each* time before running an actual/authentic sample.

12.6. Data Analysis and Calculation of Recovery and Concentration

12.6.1. To calculate the recovery (%R) of the SBMTE, use the following following formula:

$$\%R = \frac{A_u}{A_{cal}} \times 100$$

where:
A_u = Measured peak area of the *isotopically labelled* D6-SBMTE in the urine sample.
A_{cal} = Average measured peak area of the *isotopically labelled* D6-SBMTE of the calibration sample set.

12.7. Data Quantitation
12.7.1. Calculate the peak area of SBMTE in each calibration standard.
12.7.2. Calculate the peak area of D6-SBMTE internal standard in each calibration standard.
12.7.3. Create a calibration curve using the concentration of each calibrant as the x-axis and *RF* as the y-axis. Calculate *RF* as follows:

$$RF = \frac{A_{SBMTE}}{A_{IS}}$$

where:
A_{SBMTE} = Measured peak area of the SBMTE in the calibration standard.
A_{IS} = Measured peak area of the D6-SBMTE in the calibration standard.

12.7.4. Calculate RF for SBMTE in each urine sample analyzed.
12.7.5. Calculate the concentration of SBMTE in the urine sample using the calibration curve.
12.7.6. Correct the SBMTE concentration value for the concentration effect due to sample preparation. This corresponds to a factor of 2x for this sample preparation method.

13. METHOD PERFORMANCE:

13.1. Performance data and related information are provided herein only as examples and guidance. The data do not represent required performance criteria for users of the methods.
13.2. The experimentally determined LC-HRMS method figures of merit for SBMTE detection in urine are:
13.2.1. LOD: 5 ppb
13.2.2. LOQ: 13 ppb
13.2.3. Recovery for 50 ppb SBMTE spikes: 46 ± 11%
13.2.4. Recovery for 500 ppb SBMTE spikes: 81 ± 11%

14. REFERENCES

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14.5. Daly, J. D. et al. *J. Chromatogr. B* **2007**, *850*, pp. 120-127.
14.6. Halme, M. et al. *J. Chromatogr. B* **2011**, *879*, pp. 908-914.

15. POLLUTION PREVENTION

15.1. Pollution prevention encompasses any technique that reduces or eliminates the quantity and/or toxicity of waste at the point of generation. Numerous opportunities for pollution prevention exist in laboratory operation. The EPA has established a preferred hierarchy of environmental management techniques that places pollution prevention as the management option of first choice. Whenever feasible, laboratory personnel should use pollution prevention techniques to address their waste generation. When wastes cannot be feasibly reduced at the source, the Agency recommends recycling as the next best option.

15.2. For information about pollution prevention that may be applicable to laboratories and research institutions consult “Less is Better: Laboratory Chemical Management for Waste Reduction,” available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th St., N.W. Washington, D.C. 20036, http://www.acs.org.

16. WASTE MANAGEMENT

16.1. Waste suspected of contamination with chemical warfare agents and all biological waste should be neutralized before disposal. At LLNL, Document LLNL-MI-417220, “Neutralization Procedure for Chemical Agents and Toxins” should be followed. Decontamination will use commercial bleach to neutralize any agent remaining.

16.2. The Environmental Protection Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations. The Agency urges laboratories to protect the air, water, and land by minimizing and controlling all releases from hoods and bench operations, complying with the letter and spirit of any sewer discharge permits and regulations, and by complying with all solid and hazardous waste regulations, particularly the hazardous waste identification rules and land disposal restrictions. For further information on waste management, consult “The Waste Management Manual for Laboratory Personnel,” available from the American Chemical Society.